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Acid Lability of Metabolites of 2-Deoxyglucose in Rat Brain: Implications for Estimates of Kinetic Parameters of Deoxyglucose Phosphorylation and Transport Between Blood and Brain

Gerald A. Dienel, Nancy F. Cruz, Kentaro Mori, and Louis Sokoloff

Laboratory of Cerebral Metabolism, National Institute of Mental Health, U.S. Public Health Service, Department of Health and Human Services, Bethesda, Maryland, U.S.A.

Abstract: The steady-state brain/plasma distribution ratios of [^{14}C]deoxyglucose ([^{14}C]DG) for hypoglycemic rats previously determined by measurement of DG concentrations in neutralized acid extracts of freeze-blown brain and plasma exceeded those predicted by simulations of kinetics of the DG model. Overestimation of the true size of the precursor pool of [^{14}C]DG for transport and phosphorylation could arise from sequestration of [^{14}C]DG within brain compartments and/or instability of metabolites of [^{14}C]DG and regeneration of free [^{14}C]DG during the experimental period or extraction procedure. In the present study, the concentrations of [^{14}C]DG and glucose were compared in samples of rat brain and plasma extracted in parallel with perchloric acid or 65% ethanol containing phosphate-buffered saline. The concentrations of both hexoses in acid extracts of brain were higher than those in ethanol, whereas hexose contents of plasma were not dependent on the extraction procedure. The magnitude of overestimation of DG content (about 1.2- to fourfold) varied with glucose level and was highest in extracts isolated from hypoglycemic rats; contamination of the [^{14}C]DG fraction with ^{14}C-labeled nonacidic metabolites also contributed to this overestimation. Glucose concentrations in acid extracts of brain exceeded those of the ethanol extracts by <40% for normal and hypoglycemic rats. **Key Words:** Glucose metabolism—2-Deoxysugars—Perchloric acid extraction. **Dienel G. A. et al.** Acid lability of metabolites of 2-deoxyglucose in rat brain: Implications for estimates of kinetic parameters of deoxyglucose phosphorylation and transport between blood and brain. *J. Neurochem.* **54,** 1440–1448 (1990).

Many studies of the kinetics of the interconversion of 2-[^{14}C]deoxyglucose ([^{14}C]DG) and 2-[^{14}C]deoxyglucose-6-phosphate ([^{14}C]DG-6-P) and of the relative potential for phosphorylation of [^{14}C]DG and labeled glucose by hexokinase in brain in vivo (Hawkins and Miller, 1978; Miller and Kiney, 1981; Huang and Veech, 1985; Jenkins et al., 1986; Nelson et al., 1986; Bass et al., 1987; Pelligrino et al., 1987; Mori et al., 1989) or in brain slices in vitro (Newman et al., 1988*a,b*) have employed perchloric acid (PCA) to prepare the protein-free extracts from which [^{14}C]DG, [^{14}C]DG-6-P, and labeled glucose and its labeled products were separated and measured. In the course of studies on the influence of arterial plasma glucose concentration on experimentally determined steady-state brain/plasma distribution ratios for DG and glucose in brain (Mori et al., 1989), we obtained distribution ratios for DG that appeared to be inconsistent with model-predicted values, particularly in hypoglycemia (J. Holden, unpublished observations). Specifically, the measured steady-state concentrations of free [^{14}C]DG in brain were close to those predicted to serve as precursor for phosphorylation and back-transport from brain to plasma in normoglycemia and hyperglycemia, but they exceeded considerably the values predicted to occur in hypoglycemia. The [^{14}C]DG concentration measured in neutralized acid extracts of freeze-blown brain would overestimate, of

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Address correspondence and reprint requests to Dr. G. A. Dienel at Laboratory of Cerebral Metabolism, National Institute of Mental Health, Bldg. 36, Room 1A-05, 9000 Rockville Pike, Bethesda, MD 20892, U.S.A.

Abbreviations used: DG, 2-deoxy-D-glucose; DG-1-P, 2-deoxy-D-glucose-1-phosphate; DG-6-P, 2-deoxy-D-glucose-6-phosphate; GDP-DG, guanosine diphosphodeoxyglucose; glucose-6-P, glucose-6-phosphate; PCA, perchloric acid; UDP-DG, uridine diphosphodeoxyglucose.

course, the size of this precursor pool if there were the following: (a) a higher concentration of [^{14}C]DG in the extracellular than in the intracellular space; (b) some [^{14}C]DG sequestered in intracellular compartments other than the precursor pool; or (c) metabolites of [^{14}C]DG that were degraded to regenerate [^{14}C]DG either in vivo during the experimental period or during the tissue extraction and/or analytical procedures.

There is considerable evidence in the literature, beginning with reports by Emil Fischer and colleagues more than 60 years ago (Fischer et al., 1920; Bergmann et al., 1922), that *N*- and *O*-glycosides of 2-deoxysugars are much more labile to acid hydrolysis than the corresponding derivatives of the parent sugars (Overend et al., 1949; Butler et al., 1950; Fischer and Weidemann, 1964; Biely and Bauer, 1967; Schmidt et al., 1974; Lehle and Schwarz, 1976). In fact, nucleotide derivatives of 2-deoxyglucose [e.g., uridine diphosphodeoxyglucose (UDP-DG) and guanosine diphosphodeoxyglucose (GDP-DG)] are rapidly hydrolyzed to DG by 0.9 *M* PCA at 0°C (Schmidt et al., 1976). Because in our studies we had used PCA to prepare the protein-free extracts, it was possible that hydrolysis of glycosides and glycosyl phosphate esters of [^{14}C]DG was the source of the excess [^{14}C]DG. Furthermore, the low tissue concentrations of glucose and its metabolites in hypoglycemia could have led, by reduced competitive inhibition, to greater formation and proportions of these derivatives of [^{14}C]DG-6-P, which could explain the finding of greater discrepancy between measured and predicted values in hypoglycemia.

In the present studies, we have employed an alternative extraction procedure, one that avoided the use of acid, to determine if significant amounts of [^{14}C]DG were regenerated when acid was used to obtain protein-free extracts of brain labeled in vivo with [^{14}C]DG. The results demonstrated higher concentrations of [^{14}C]DG, and to a lesser extent also glucose, in neutralized PCA extracts than in extracts obtained by parallel extraction of portions of the same brain samples with 65% ethanol in phosphate-buffered saline. The magnitude of the overestimations in acid extracts varied with the tissue glucose level and was greatest in brain from hypoglycemic rats.

MATERIALS AND METHODS

Animals

The plasma and freeze-blown brain samples analyzed in the present study were obtained from the same animals (normal male Sprague–Dawley rats, weighing 250–350 g; Taconic Farms, Germantown, NY, U.S.A.) used in our previous study (Mori et al., 1989); they had been stored at −80°C. Briefly, the rats were administered programmed intravenous infusions of [^{14}C]DG designed to produce and maintain a constant arterial plasma concentration while the arterial plasma glucose concentration was clamped at a constant level between about 2 and 28 m*M*. After the arterial hexose concentrations had been maintained relatively constant for 45 min for [^{14}C]DG and 75–90 min for glucose, long enough for steady states between brain and plasma to have been achieved for both hexoses, the brains were removed by freeze-blowing (Veech et al., 1973).

Tissue extraction

The frozen brains were powdered under liquid N_2 in a cryostat maintained at about −35°C and divided into weighed 100–200-mg portions. Samples were deproteinized by thawing in 3 volumes of 3 *M* PCA at −12°C, followed by dilution of the acid to 0.6 *M* and centrifugation to remove the precipitated protein. Samples (50 μl) of plasma obtained from the animal approximately simultaneously with the brain tissue were diluted with equal volumes of water and deproteinized by addition of 1.0 ml of 0.7 *M* PCA followed by centrifugation. All acid extracts were neutralized to pH 7.0–7.5 with 2.0 *M* KOH/0.4 *M* imidazole/0.4 *M* potassium acetate within 1 h after addition of the acid (Lowry and Passonneau, 1972), and the $KClO_4$ precipitates were removed by centrifugation. The extracts were stored at −80°C until analyzed.

Portions of the same brains were also extracted in parallel with ethanol by a modification of the procedure of Schmidt et al. (1974). Brain powders were extracted serially with ethanol/phosphate-buffered saline/water (65:10:25, by volume). The saline solution consisted of 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, and 0.2 g of KH_2PO_4 dissolved in 800 ml of water (Dulbecco and Vogt, 1954). The ice-cold ethanol solution (2 ml) was slowly added to the frozen brain powder (100–200 mg) in a tube kept on dry ice, and the slurry was mixed by gentle vortexing. The tube was then transferred to an alcohol bath maintained at −8° to −10°C, and the slurry was mixed until the ethanol solution penetrated the tissue powder. Remaining lumps of powder were dispersed with a glass rod which was rinsed with 1 ml of ethanol solution. The slurry was mixed periodically for about 15 min at 0°C. The sample was centrifuged at 6,000 *g* for 30 min at 2–4°C, the supernatant was transferred to another tube, and the pellet was resuspended by stirring with a glass rod and reextracted as above three more times. The combined supernatant fractions were lyophilized in a SpeedVac Concentrator (Savant Instruments, Farmingdale, NY, U.S.A.). This drying step was monitored very carefully; each sample was removed from the concentrator and dissolved in 1.0–2.0 ml of ice-cold water as soon as possible after it was dry, because preliminary evidence suggested that warming of the samples in the dry state could cause derivatization of hexoses. The resulting turbid solution was clarified by centrifugation at 31,000 *g* for 60 min at 2–4°C, and the supernatant fraction was rapidly frozen in dry ice and stored at −80°C. Diluted plasma samples (100 μl) were extracted by addition of ice-cold ethanol/saline solution (700 μl), mixing at 0°C for 15 min, centrifugation at 6,000 *g* for 30 min at 2–4°C, and lyophilization of the supernatant solutions as above. These supernatant fractions were dissolved in 1.0 ml of water, quickly frozen in dry ice, and stored at −80°C. All ethanol extracts of brain and plasma were assayed for their hexose contents within 3 days of extraction. The glucose contents and the fraction of total ^{14}C in [^{14}C]DG in the ethanol extracts of brain were found not to change significantly with storage at −80°C for at least 6 months and two freeze-thawing cycles.

Measurement of glucose and [^{14}C]DG concentrations

Samples of unextracted plasma and of neutralized PCA or ethanol extracts of brain and plasma were incubated with 50 m*M* Tris, pH 8.3, 17 m*M* $MgCl_2$, 8 m*M* ATP, and 4 units (SI) of yeast hexokinase (Boehringer, Indianapolis, IN,

U.S.A.) to derivatize [^{14}C]DG to [^{14}C]DG-6-P; enzyme-free reaction mixtures were incubated in parallel with each sample. Portions (0.5 ml) of these incubation mixtures or of the neutralized acid extracts of plasma and brain that had not been incubated as above were applied to 2 ml of Dowex-AG1-X8-formate columns (100–200 mesh, 0.8 × 5 cm; Bio-Rad, Richmond, CA, U.S.A.). The columns were washed with 23 ml of water and eluted with 23 ml of 2 *M* HCl/2 *M* NaCl. Samples of the column effluents and eluates were assayed for ^{14}C by liquid scintillation counting (Beckman Model LS 5801, Beckman Instruments, Fullerton, CA, U.S.A.) with external standardization or internal standardization with [^{14}C]toluene as the internal standard. The Dowex-1-formate column effluents derived from the samples that had not been derivatized with ATP and hexokinase contained the total ^{14}C in the nonacidic compounds, including the [^{14}C]DG; the corresponding effluents isolated after derivatization of [^{14}C]DG to [^{14}C]DG-6-P contained the ^{14}C-labeled nonacidic metabolites of [^{14}C]DG. The [^{14}C]DG concentration of each sample, therefore, was calculated by subtracting the nonphosphorylatable ^{14}C from the total in the nonacidic fraction.

Glucose contents of the neutralized acid and the ethanol extracts were measured enzymatically with hexokinase and glucose-6-phosphate (glucose-6-P) dehydrogenase (Lowry and Passonneau, 1972). Glucose in the unextracted plasma samples was measured by the glucose oxidase method in a Beckman Glucose Analyzer II (Beckman Instruments).

Recoveries of DG and glucose with the ethanol extraction procedure were assessed as follows. [6-^{14}C]Glucose and 2-[1-^{14}C]DG (sp. act. 55 and 49 mCi/mmol, respectively; New England Nuclear Corp., Boston, MA, U.S.A.) were added to frozen powders of nonradioactive freeze-blown brain and extracted with ethanol as above. Approximately 97% of the added ^{14}C was recovered in the ethanol extracts (see Table 3). A fraction of the extract was chromatographed on a CarboPac PA1 anion-exchange column with a Dionex BioLC HPLC system equipped with a Dionex pulsed amperometric detector (Dionex Corp., Sunnyvale, CA, U.S.A.). The following elution gradient was used: isocratic at 5 m*M* NaOH for 15 min, 15 m*M* NaOH from 15.1 to 20 min, and 30 m*M* NaOH from 20.1 to 25 min; a linear gradient of 100 m*M* NaOH/300 m*M* sodium acetate to 100 m*M* NaOH/600 m*M* sodium acetate from 25.1 to 55 min; isocratic at 200 m*M* NaOH from 55.1 to 75 min. Hexose and hexose-6-phosphate standards were used to calibrate the elution times for the appropriate monosaccharides and their derivatives. Samples of the eluates were collected at 1-min intervals, neutralized with glacial acetic acid, and assayed for their ^{14}C contents by liquid scintillation counting.

RESULTS

Comparison of recoveries of [^{14}C]DG and ^{14}C-labeled metabolites in PCA and ethanol extracts of brain

Unmetabolized [^{14}C]DG in brain is usually assayed in the nonacidic fractions (i.e., effluents from anion-exchange columns) of acid extracts of the tissue, and the total ^{14}C in these fractions is often assumed to represent the [^{14}C]DG content of the tissue (Huang and Veech, 1985; Jenkins et al., 1986; Nelson et al., 1986; Bass et al., 1987; Pelligrino et al., 1987; Newman et al., 1988*a,b*; Mori et al., 1989). In the present studies, however, we recovered significantly greater amounts of ^{14}C in the Dowex-1-formate column effluents of neutralized acid extracts than of ethanol extracts of paired samples of the same brains (Tables 1 and 2). When the [^{14}C]DG in these nonacidic fractions was selectively derivatized to [^{14}C]DG-6-P with hexokinase

TABLE 1. *Influence of extraction procedure on recovered concentrations of glucose and of [^{14}C]DG and its ^{14}C-labeled metabolites in brain*

Plasma glucose level (m*M*)	Extraction procedure	^{14}C-Labeled compounds in brain (nCi/g)			Brain glucose concentration (μmol/g)
		Nonacidic compounds		Acidic metabolites	
		Total	DG		
Hypoglycemia:					
2.3 ± 0.1	PCA	86 ± 10[a]	—	938 ± 118	0.15 ± 0.01[a]
	EtOH	25 ± 3	17 ± 3	920 ± 125	0.12 ± 0.02
Normoglycemia:					
9.0 ± 1.7	PCA	83 ± 12[a]	—	250 ± 84	2.05 ± 0.50[a]
	EtOH	63 ± 7	60 ± 7	251 ± 69	1.82 ± 0.51
Hyperglycemia:					
22.7 ± 3.1	PCA	51 ± 10[a]	—	80 ± 18	4.96 ± 0.40
	EtOH	44 ± 8	42 ± 8	83 ± 17	4.77 ± 0.57

The values are the means ± SD of determinations in six animals. Portions of each brain were extracted with PCA or with 65% ethanol containing phosphate-buffered saline (EtOH) and assayed for total nonacidic compounds and acidic ^{14}C-labeled products by anion-exchange column chromatography on Dowex-1-formate and liquid scintillation counting. [^{14}C]DG was assayed in the nonacidic fractions by hexokinase-catalyzed phosphorylation and separation on Dowex-1-formate; glucose was assayed enzymatically in the total extracts (see Materials and Methods). A dash (—) denotes that the volumes of the samples were insufficient for derivatization of their [^{14}C]DG contents with hexokinase and analysis of their contents of [^{14}C]DG and nonacidic ^{14}C-metabolites. Therefore, paired brain samples from five additional rats with a range of arterial plasma glucose concentrations were extracted in parallel with PCA or ethanol and analyzed for [^{14}C]DG and nonacidic ^{14}C-labeled metabolites; these results are presented in Table 2.

[a] Significantly greater than the concentrations in ethanol-extracted samples ($p < 0.001$, paired t test).

TABLE 2. *Effect of extraction procedure on concentrations of [^{14}C]DG and nonacidic ^{14}C-labeled metabolites in brain*

Plasma glucose level (m*M*)	Extraction procedure	^{14}C-Labeled compounds (nCi/g)				Brain glucose concentration (μmol/g)
		Nonacidic compounds			Acidic metabolites	
		Total	DG	Metabolites		
3.2	PCA	68	63	5	618	0.21
	EtOH	27	22	5	643	0.15
4.0	PCA	62	58	4	397	0.77
	EtOH	37	34	3	385	0.59
5.9	PCA	46	44	2	216	1.20
	EtOH	32	30	2	203	1.08
12.0	PCA	90	88	2	237	2.64
	EtOH	72	69	3	224	2.56
13.2	PCA	82	78	4	232	2.76
	EtOH	62	58	4	221	2.61

The values are those obtained in individual rats at each plasma glucose level. Portions of each brain of five individual rats with a range of plasma glucose levels were extracted with PCA or with 65% ethanol containing phosphate-buffered saline (EtOH), as described in the legend to Table 1 and Materials and Methods. The extracts were assayed for glucose, [^{14}C]DG, and acidic and nonacidic ^{14}C-labeled metabolites of [^{14}C]DG by hexokinase-catalyzed phosphorylation and chromatography on Dowex-1-formate columns, as described in Materials and Methods. The concentrations of the total ^{14}C in the nonacidic fractions (i.e., Dowex-1-formate column effluents), [^{14}C]DG, and glucose were all significantly greater in the PCA extracts than in the ethanol extracts ($p < 0.05$, paired t test); the contents of nonacidic and acidic metabolites were, however, not significantly different in the PCA and ethanol extracts.

and then separated by Dowex-1-formate column chromatography, significantly ($p < 0.05$) greater amounts of ^{14}C were recovered in the derivatized fractions from the acid extracts than from the ethanol extracts (Table 2). It was clear that the recoveries of [^{14}C]DG were greater with acid extraction than with ethanol extraction of brain tissue.

The lower recoveries of [^{14}C]DG in the ethanol extracts compared to the acid extracts were due neither to incomplete extraction of [^{14}C]DG nor to derivatization of [^{14}C]DG to other compounds by enzymatic (e.g., to [^{14}C]DG-6-P by hexokinase) or nonenzymatic reactions during the extraction procedure. They were due instead to degradation of acid-labile metabolites of [^{14}C]DG during the acid extraction procedure. Virtually all of [^{14}C]DG added to a nonradioactive brain was recovered in the ethanol extracts as unaltered [^{14}C]DG when separated and assayed by HPLC (Table 3). When ethanol extracts were exposed to 0.6 *M* PCA for 30 min at 0°C, however, the recoveries of ^{14}C in the nonacidic fractions clearly exceeded those of other samples of the same extracts not treated with acid (Table 4). This difference between the acid-treated and untreated ethanol extracts varied with the plasma glucose level; the difference was greatest in hypoglycemia (Table 4). After acid treatment, the contents of the nonacidic ^{14}C-labeled compounds rose to within 10% of those found in PCA extracts of portions of the same brains (Table 4). The acidification of the ethanol extracts, therefore, must have degraded acid-labile ^{14}C-labeled metabolites to regenerate [^{14}C]DG (see Tables 1 and 2). The stabilities of these acid-labile metabolites during Dowex-1-formate column chromatography were not tested, but significant degradation during the water washes of the columns to recover the nonacidic compounds would have attenuated the differences between the PCA and ethanol extracts. Acid-catalyzed degradation of these metabolites would be expected to occur when the acidic metabolites were eluted from the columns with acid, but the ^{14}C would then still have been recovered in the proper fraction (i.e., acidic metabolites).

TABLE 3. *Recovery of ^{14}C-hexoses added to nonradioactive brain by the ethanol extraction procedure*

Fraction	Percent recovery of ^{14}C	
	[6-^{14}C]Glucose (n = 3)	[1-^{14}C]DG (n = 4)
Total ethanol extract	96.8 ± 0.4	97.4 ± 0.7
Fractions separated by HPLC		
Hexose fraction	94.8 ± 1.9	98.1 ± 2.4
Hexose-6-phosphate fraction	1.3 ± 0.7	0.6 ± 0.1
Other fractions	3.4 ± 2.0	0.4 ± 0.5

The values are means ± SD of the number of experiments in parentheses. ^{14}C-Hexoses were added to portions of frozen powders of unlabeled freeze-blown brain. The samples were extracted with 65% ethanol containing phosphate-buffered saline (see Materials and Methods), and portions of the extracts were assayed for total ^{14}C by liquid scintillation counting. The ^{14}C-labeled compounds in other portions of the ethanol extracts were then separated by HPLC with a Dionex BioLC system. Fractions were collected from the column at 1-min intervals and assayed for their ^{14}C contents by liquid scintillation counting (see Materials and Methods). Recovery of total ^{14}C applied to the column averaged 98.8 ± 1.9% (mean ± SD, n = 15).

TABLE 4. *Recovery of ^{14}C in nonacidic compounds after acidification of the ethanol extract of brain*

Rat no.	Plasma glucose level (m*M*)	Extraction procedure	Nonacidic ^{14}C-compounds (nCi/g)
1	2.5	PCA	91
		EtOH	
		Before acidification	27
		After acidification	82
2	5.6	PCA	42
		EtOH	
		Before acidification	26
		After acidification	40
3	8.9	PCA	75
		EtOH	
		Before acidification	57
		After acidification	67
4	22.2	PCA	51
		EtOH	
		Before acidification	42
		After acidification	48

Portions of each brain of four individual rats, which were given continuous infusions of glucose and [^{14}C]DG to produce and maintain constant arterial concentrations of these hexoses, were extracted with PCA or with 65% ethanol containing phosphate-buffered saline (EtOH), as described in the legends to Tables 1 and 2 (see Materials and Methods). Portions of each EtOH extract were acidified with PCA (0.6 *M* final concentration), kept on ice for 30 min, neutralized, and centrifuged to remove the $KClO_4$. All samples were then assayed for total ^{14}C-labeled nonacidic compounds by anion-exchange column chromatography on Dowex-1-formate and liquid scintillation counting (see Materials and Methods).

Nonphosphorylatable ^{14}C in the nonacidic fraction (i.e., ^{14}C not retained by Dowex-1-formate after incubation with hexokinase and ATP) was assumed to represent nonacidic ^{14}C-labeled metabolites derived from [^{14}C]DG. The amounts of nonphosphorylatable ^{14}C recovered from the acid and ethanol extracts of brain were not statistically significantly different (Table 2).

Most of the ^{14}C in the acid and ethanol extracts of brain was recovered in the acidic metabolites of [^{14}C]DG (i.e., ^{14}C in total brain extracts retained on Dowex-1-formate columns and then eluted and assayed) (Tables 1 and 2). The recoveries of the ^{14}C-labeled metabolites from the acid and ethanol extracts were not statistically significantly different (Tables 1 and 2).

Influence of plasma glucose level on recoveries of [^{14}C]DG and ^{14}C-labeled metabolites in PCA and ethanol extracts of brain

The arterial plasma glucose concentration had major influences on the distribution of ^{14}C between the acidic and nonacidic fractions of both the PCA and ethanol extracts of brain. With decreasing arterial plasma glucose concentration, brain glucose concentration decreased, the fraction of total ^{14}C in the brain extracts recovered in the ^{14}C-labeled acidic metabolites increased, and the [^{14}C]DG contents of the brain decreased (Tables 1 and 2). These findings were similar with both acid and ethanol extraction of brain and reflected the effects of more rapid phosphorylation of [^{14}C]DG by hexokinase because of reduced competitive inhibition associated with the lower glucose concentrations in the brain.

The magnitude of the discrepancies between the amounts of [^{14}C]DG and total ^{14}C recovered in the nonacidic fractions of PCA extracts and of ethanol extracts of brain was also influenced by the arterial plasma glucose concentration. The discrepancies were considerably greater in hypoglycemic rats than in normoglycemic and hyperglycemic rats (Tables 1, 2, and 4; Fig. 1). For example, the ratios of total ^{14}C and [^{14}C]DG recovered from the acid extracts to the corresponding recoveries from the ethanol extracts increased very moderately with decreasing arterial plasma glucose levels within the normoglycemic range (e.g., 6–13 m*M*), but rose sharply when the plasma glucose level was reduced below 4 m*M* (Table 2, Fig. 1).

Nearly all the ^{14}C in the nonacidic fractions of both the acid extracts (Table 2) and ethanol extracts (Tables 1 and 2) from the brains of normoglycemic and hyperglycemic rats was recovered in the derivatized [^{14}C]DG. On the other hand, the total ^{14}C recovered in the nonacidic fractions of both the acid and ethanol extracts of the brains from the hypoglycemic rats exceeded the ^{14}C recovered in the [^{14}C]DG (Tables 1 and 2). These excesses, which represented nonacidic ^{14}C-

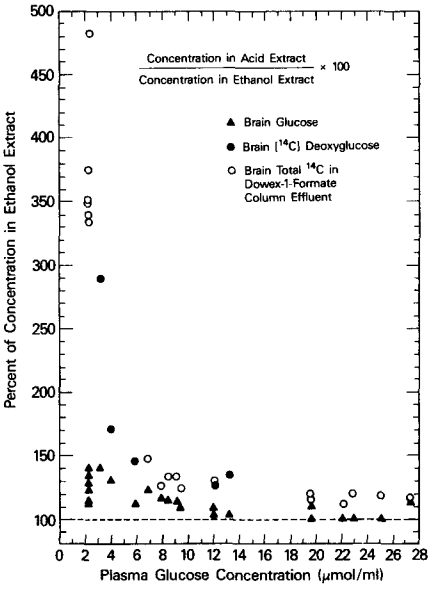

FIG. 1. Magnitude of overestimation of concentrations of [^{14}C]DG and glucose in PCA extracts of brain as a function of the arterial plasma glucose concentration. The values correspond to either the total ^{14}C (○) or the [^{14}C]DG (i.e., fraction phosphorylatable by hexokinase) (●) recovered in the Dowex-1-formate effluents; the glucose concentrations (▲) were measured enzymatically in the extracts (Tables 1 and 2).

labeled metabolites of [^{14}C]DG in brain, did not differ significantly between the acid and ethanol extracts (Table 2). The formation of nonacidic derivatives of [^{14}C]DG in brain appeared to be enhanced with increasing severity of hypoglycemia [i.e., 2.0 ± 0.9, 3.1 ± 0.4, and 8.3 ± 1.0 nCi/g (means ± SD) when the plasma glucose levels averaged 22.7, 9.0, and 2.3 m*M*, respectively; see Table 1].

Recoveries of glucose in PCA and ethanol extracts of brain

The glucose concentrations recovered in the brains of the normoglycemic and hypoglycemic rats were significantly greater in the acid extracts than in the ethanol extracts (Tables 1 and 2), and the magnitude of the difference was also dependent on the arterial plasma glucose concentration (Fig. 1). The percent difference between the glucose recoveries in the PCA and ethanol extracts rose progressively to a maximum of about 40% as the arterial plasma glucose level was reduced from about 12 to 2 m*M* (Fig. 1). This difference was not due to incomplete extraction of glucose, significant loss of glucose during the ethanol extraction procedure, or interferences with the glucose assay. When the ethanol extract of brain was tested for the possibility of residual enzyme activities that might interfere with the glucose assay (e.g., NADPH oxidase, glucose-6-P dehydrogenase, or hexokinase), none was detected in the extract. Glucose-6-P or glucose standards added to ethanol extracts of brain produced the same increase in NADPH fluorescence as standards assayed in parallel without brain extract. Nearly all (95%) of the [^{14}C]glucose added to a nonradioactive powder of freeze-blown brain and subjected to ethanol extraction was recovered as glucose; 1% of the [^{14}C]glucose may have been derivatized to ^{14}C-labeled compounds that eluted with glucose-6-P and 3% to other unidentified compounds (Table 3). The fact that the recoveries of glucose were essentially the same in both the ethanol and acid extracts of the brains of the hyperglycemic rats (Tables 1 and 2) also indicates that the higher recoveries in the acid extracts than in the ethanol extracts of the brains of normoglycemic and hypoglycemic rats were not due to incomplete extraction by the ethanol.

Recoveries of glucose, [^{14}C]DG, and ^{14}C-labeled metabolites in PCA and ethanol extracts of plasma

If the higher recoveries of [^{14}C]DG and glucose in PCA extracts than in ethanol extracts of brain were due to the degradation of acid-labile metabolites back to free [^{14}C]DG or glucose during the extraction procedure rather than to more complete extraction, then such differential recoveries in acid and ethanol extracts should be absent or much less in plasma, which could be expected to have lower contents of such acid-labile metabolites. These were, indeed, the experimental findings. The concentrations of glucose, [^{14}C]DG, and nonacidic ^{14}C-labeled metabolites in plasma varied with the arterial plasma glucose level, but did not differ significantly in unextracted, PCA-extracted, and ethanol-extracted samples of the same plasma assayed in parallel (Table 5).

DISCUSSION

The present studies demonstrate that PCA extraction, a commonly used procedure to extract intermediary metabolites from tissue, leads to overestimation of [^{14}C]DG and glucose concentrations in brain. The degree of overestimation varies with the glucose levels in plasma and brain; it increases with decreased plasma and brain glucose concentrations. When glucose levels in plasma and tissue are low, competitive inhibition by glucose of [^{14}C]DG transport from blood to brain and phosphorylation by hexokinase is reduced, and [^{14}C]DG-6-P synthesis is accelerated. Also, in hypoglycemia, the levels of glucose metabolites are reduced,

TABLE 5. *Influence of extraction procedure on recovered concentrations of glucose, [^{14}C]DG, and ^{14}C-labeled metabolites of [^{14}C]DG in plasma*

Rat no.	Extraction procedure	Plasma glucose level (m*M*)	Plasma [^{14}C]DG concentration (nCi/ml)	Nonacidic ^{14}C-metabolites (nCi/ml)
1	None	2.1	55	17
	PCA	2.3	65	12
	EtOH	2.2	58	17
2	None	2.4	91	9
	PCA	2.1	86	10
	EtOH	2.1	82	10
3	None	3.0	71	19
	PCA	3.0	67	20
	EtOH	2.7	68	19
4	None	8.3	121	13
	PCA	7.9	122	13
	EtOH	7.8	119	13
5	None	8.3	168	2
	PCA	8.4	173	3
	EtOH	8.3	169	3
6	None	9.3	134	3
	PCA	9.1	131	5
	EtOH	9.2	126	4
7	None	20.3	138	16
	PCA	19.5	142	15
	EtOH	21.0	140	18
8	None	25.5	102	2
	PCA	26.9	105	3
	EtOH	25.1	109	2

Portions of each plasma sample were assayed for their concentrations of glucose, [^{14}C]DG, and ^{14}C-labeled nonacidic and acidic metabolites of [^{14}C]DG in native plasma and in PCA and ethanol (EtOH) extracts, as described in Tables 1 and 2 and Materials and Methods. The concentrations of glucose, [^{14}C]DG, and the ^{14}C-labeled metabolites in the PCA and EtOH extracts did not differ significantly from each other nor from those in native unextracted plasma ($p > 0.05$, paired t test). Small amounts of acidic ^{14}C-labeled compounds were detected in plasma (data not shown), but these accounted for only about 1.5% of the total ^{14}C content of the plasma.

further metabolism of [^{14}C]DG-6-P and/or its products is disinhibited, and entry of ^{14}C into subsequent metabolic pools and pathways is enhanced. The amounts of nonacidic and acidic ^{14}C-labeled derivatives of [^{14}C]DG found in brain are then increased, for example, as much as three- to fourfold when the plasma glucose level was reduced from a normal level of approximately 9 m*M* to 2 m*M* (Table 1).

The predominant acidic metabolite, [^{14}C]DG-6-P, is a phosphate ester that is stable to acid hydrolysis (Biely and Bauer, 1967). Almost all other products of [^{14}C]DG metabolism are derived from [^{14}C]DG-6-P; these include 2-[^{14}C]deoxyglucose-1-phosphate ([^{14}C]DG-1-P), UDP-[^{14}C]DG, GDP-[^{14}C]DG, and [^{14}C]DG incorporated into polysaccharide and oligosaccharide chains in glycogen, glycolipids, and glycoproteins (Biely and Bauer, 1967; Schmidt et al., 1974, 1976; Lehle and Schwarz, 1976; Nelson et al., 1984). Most of these secondary products possess glycosidic or glycosyl phosphate ester linkages involving the anomeric hydroxyl group of DG. Glycosides and glycosyl phosphates of 2-deoxysugars are partially to completely hydrolyzed within 20 min in weak acid (0.01–0.5 *M*) at 15–100°C, and the rates of hydrolysis increase with increasing acidity and/or temperature. Sensitivity of these glycosides to acid hydrolysis is greater for the pentose sugar, N-linkage, or furanose ring structure compared to those of the hexose, O-linkage, or pyranose structure (Bergmann et al., 1922; Overend et al., 1949; Butler et al., 1950; Fischer and Weidemann, 1964; Biely and Bauer, 1967; Zemek et al., 1971; Schmidt et al., 1974, 1976; Lehle and Schwarz, 1976). Fission of the carbon–oxygen bond to form a carbonium ion intermediate is enhanced by removal of the inductive effect of the hydroxyl group at carbon 2; addition of an electronegative substituent at carbon 2, as in 2-fluoro-2-deoxyglucose, confers resistance to acid hydrolysis (Fisher et al., 1920; Newth et al., 1947; Overend et al., 1949, 1962; Shafizadeh, 1958; Armour et al., 1961; Marshall, 1963). UDP-DG and GDP-DG are quantitatively hydrolyzed to DG within less than 5 min in 0.9 *M* PCA at 0°C, and UDP-2-deoxyhexoses and 2-deoxyhexose-1-phosphates are hydrolyzed even at pH 7 and pH 4, respectively, when heated at 100°C for 7 min (Fischer and Weidemann, 1964; Biely and Bauer, 1967; Schmidt et al., 1976). Therefore, [^{14}C]DG should be expected to be liberated from glycosides and glycosyl phosphates of [^{14}C]DG during extraction of freeze-blown brain with PCA at concentrations varying between 0.6 and 3 *M*. [^{14}C]DG may also be released by such hydrolytic reactions with microwave fixation of brain, during which brain temperatures rise to about 90°C within seconds (Medina et al., 1980), or with sonification of tissue samples without adequate cooling or inactivation of enzymes (Schmidt et al., 1974).

The acidic metabolites are obvious sources of acid-labile compounds (e.g., [^{14}C]DG-1-P, UDP-[^{14}C]DG) from which free [^{14}C]DG could be generated during PCA extraction. The recovery of acidic metabolites of [^{14}C]DG in the ethanol extracts of brain were not, however, significantly greater than those from the acid extracts (Table 1). It should be noted, however, that most of the recovered acidic metabolites consist of [^{14}C]DG-6-P, and even if the phosphoglucomutase reaction were in equilibrium, the [^{14}C]DG-1-P concentration would reach only a small fraction (5–10%) of the [^{14}C]DG-6-P concentration (Együd and Whelan, 1963). A loss of 5%, for example, from the total acidic metabolite pool because of acid hydrolysis would be difficult to detect, but the [^{14}C]DG released would increase the size of the [^{14}C]DG pool by about 20% in normoglycemia or two- to threefold in hypoglycemia. We are currently attempting to identify the acid-labile ^{14}C-labeled metabolites in the ethanol extracts of brain by Dionex BioLC HPLC of the extracts before and after acidification; preliminary results indicate that two of the acid-labile ^{14}C-labeled metabolites have the same elution times as DG-1-P and UDP-DG and regenerate [^{14}C]DG when degraded by acid.

Glycogen and oligosaccharides are likely sources of glucose derived from acid hydrolysis. Glycogen is not completely depleted from the brain in rats subjected to acute insulin-induced hypoglycemia without seizures (Ferrendelli and Chang, 1973; Lewis et al., 1974; Ghajar et al., 1982), as was the case in the present experiments. Glucosidic bonds are slowly hydrolyzed under conditions like those in the PCA extraction procedure, and glucosidic linkages vary in their sensitivity to acid hydrolysis (Pazur, 1970). Glycogen is partially degraded to polymers with 40% lower molecular weight within 2 h by 5% trichloroacetic acid at 0°C (Stetten et al., 1956, 1958). The glucose concentration in brain is relatively stable with acid extraction compared to that of [^{14}C]DG (Fig. 1), but some small increases in glucose concentration did occur with acid extraction, particularly in the hypoglycemic animals.

PCA extraction of the brain tissue has been commonly used in previous biochemical studies of the distribution of ^{14}C between [^{14}C]DG and [^{14}C]DG-6-P following a pulse of [^{14}C]DG. The artifactual expansion of the apparent [^{14}C]DG pool by hydrolysis of labeled metabolites during the extraction procedure would lead, of course, to misleading results. The magnitude of the errors would increase with increasing severity of hypoglycemia, and also with increasing time after the pulse of the tracer as the acid-labile ^{14}C-labeled metabolites accumulate while the [^{14}C]DG is cleared from the tissue. This artifact may explain, at least in part, the extremely high brain/blood distribution ratios for [^{14}C]DG (e.g., 0.9 and 3.0 at 45 and 240 min after the pulse, respectively) reported by Hawkins and Miller (1978), the slowly decaying pool of acid-extractable [^{14}C]DG reported by Newman et al. (*1988a*) in brain slices in vitro, and our recent observation of steady-state brain/plasma distribution ratios for [^{14}C]DG that exceeded 1.0 in severely hypoglycemic rats (Mori et al., 1989). We are currently reanalyzing the hexose composition of samples of plasma and brain remaining

from the study of Mori et al. (1989) by extracting them with 65% ethanol, as in the present studies. The results thus far indicate that acid-labile metabolites of [^{14}C]DG are present in both the soluble and insoluble fractions of the ethanol extracts of brain and that nonacidic labeled metabolites with the same elution times as 2-deoxysorbitol and 2-deoxygalactose have been separated from [^{14}C]DG with the Dionex BioLC Chromatography System.

The enhancement of the precursor [^{14}C]DG pool in brain by hydrolysis of acid-labile products during PCA extraction may also have played a role in a number of studies that attempted to determine the time courses of [^{14}C]DG and [^{14}C]DG-6-P concentrations in brain after a pulse of [^{14}C]DG and to estimate from them the relative rates of [^{14}C]DG phosphorylation and [^{14}C]DG-6-P dephosphorylation (Hawkins and Miller, 1978; Miller and Kiney, 1981; Huang and Veech, 1985; Jenkins et al., 1986; Nelson et al., 1986; Bass et al., 1987; Pelligrino et al., 1987; Newman et al., 1988*a,b*). The size of the precursor pool of [^{14}C]DG in brain must have been overestimated in all of these studies by a magnitude that would vary with the time after the pulse and the plasma and brain glucose concentrations. The overestimation would increase with duration of the experiment as the acid-labile ^{14}C-labeled metabolites accumulate in the brain and the [^{14}C]DG is cleared. The inflation of the size of the [^{14}C]DG pool in brain in those experiments with the pulse would be even greater than that observed in the normoglycemic animals in the present studies in which the [^{14}C]DG content of the tissue did not decline with time, but was maintained constant by a programmed infusion (Mori et al., 1989). For example, at 45 min, the [^{14}C]DG content in normoglycemic rats is about 20–25% of the total ^{14}C in the brain during a square-wave input, but only about 10% after a pulse; the overestimation at this time was 30–50% with the square-wave (Table 2) and could be expected to be even more after a pulse because of the low true levels of [^{14}C]DG in the brain at that time. The theoretical rates of [^{14}C]DG-6-P formation computed from the artifactually inflated values for free [^{14}C]DG would exceed the measured rates of [^{14}C]DG-6-P accumulation and lead to erroneous overestimation of the degree of dephosphorylation of [^{14}C]DG-6-P (Huang and Veech, 1985; Bass et al., 1987; Pelligrino et al., 1987).

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